

# Defective Angiogenesis in the Inflammatory Granulation Tissue in Histidine Decarboxylase-deficient Mice but not in Mast Cell-deficient Mice

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## Abstract

We have analyzed the role of histamine in the angiogenesis of the granulation tissue in histidine decarboxylase-deficient (HDC<sup>-/-</sup>) mice, mast cell-deficient mice (WBB6F1-*W/W<sup>v</sup>*), and their corresponding wild-type mice (HDC<sup>+/+</sup> and WBB6F1<sup>+/+</sup>). In HDC<sup>+/+</sup> mice, subcutaneous implantation of a cotton thread in the dorsum induced granulation tissue formation with angiogenesis, while the topical injection of antivascular endothelial growth factor (VEGF) IgG strongly suppressed them. In HDC<sup>-/-</sup> mice which showed lower VEGF levels in the granulation tissue, there was notably less angiogenesis and granulation tissue formation than in HDC<sup>+/+</sup> mice. The topical injection of histamine or the H<sub>2</sub> agonist dimaprit rescued the defective angiogenesis and granulation tissue formation in HDC<sup>-/-</sup> mice. There was no significant difference in the granulation tissue formation and angiogenesis between WBB6F1-*W/W<sup>v</sup>* and WBB6F1<sup>+/+</sup> mice. In addition, macrophages in the granulation tissue were found to express HDC. Our findings indicate that histamine derived from nonmast cells plays a significant role in the angiogenesis of the inflammatory granulation tissue.

Key words: cotton thread • dimaprit • histamine • macrophage • vascular endothelial growth factor

## Introduction

The progress of chronic and proliferative inflammation depends on angiogenesis (1). It is required not only for the maintenance of tissue perfusion, but also to allow an increase in the cellular traffic required for chronicity (2). Therefore, the inhibition of angiogenesis would be a valid target for drug development in chronic inflammatory diseases such as rheumatoid arthritis (3), atherosclerosis (4), diabetic retinopathy (5), psoriasis (6), wound healing (7), and chronic airway inflammation (8), as well as for antineoplastic therapy and Kaposi's sarcoma (9).

Vascular endothelial growth factor (VEGF)\* is a potent inducer of angiogenesis (10). Hypoxia conditions induce VEGF production, in which the transcription factor hypoxia-inducible factor-1 is activated (11). In addition,

PGE<sub>2</sub> induces VEGF production via the cAMP-protein kinase A pathway (12) and enhances angiogenesis in carrageenin-induced proliferative granulation tissue (13) and in cultures of human umbilical vein endothelial cells treated with the conditioned medium of colon cancer cells (14). However, we have suggested that mediators other than PGE<sub>2</sub> participate in VEGF production by the inflammatory granulation tissue, because the cyclooxygenase inhibitors indomethacin and NS-398 only partially suppressed the VEGF production (13). Zauberman et al. (15) first showed that histamine is an angiogenic in rabbit cornea. In addition, histamine was found to be produced in rapidly growing granulation tissue (16, 17) and was thought to promote neoplastic growth and angiogenesis (18–21). It is also reported that endogenous histamine has dual roles in angiogenesis in rats (22). Although these findings indicate that histamine might have the ability to induce angiogenesis, convincing proof that endogenous histamine upregulates angiogenesis in the granulation tissue is still lacking. Recently, we found that in a carrageenin-induced air pouch-type inflammation model in rats, the angiogenesis that occurs

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\*Abbreviations used in this paper: HDC, histidine decarboxylase; VEGF, vascular endothelial growth factor.

during the formation of granulation tissue was inhibited by treatment with cimetidine through the suppression of VEGF protein induction (23). In addition, *in vitro* experiments revealed that histamine induces VEGF production in the granulation tissue (23). However, the specific role of histamine in angiogenesis and the source of histamine in the proliferative inflammatory tissue remain to be elucidated.

In this study, we analyzed the role of endogenous histamine in the angiogenesis of the cotton thread-induced granulation tissue using histidine decarboxylase-deficient mice (HDC<sup>-/-</sup>), mast cell-deficient mice (WBB6F1-*W/W<sup>v</sup>*), and their corresponding wild-type mice (HDC<sup>+/+</sup> and WBB6F1<sup>+/+</sup>).

## Materials and Methods

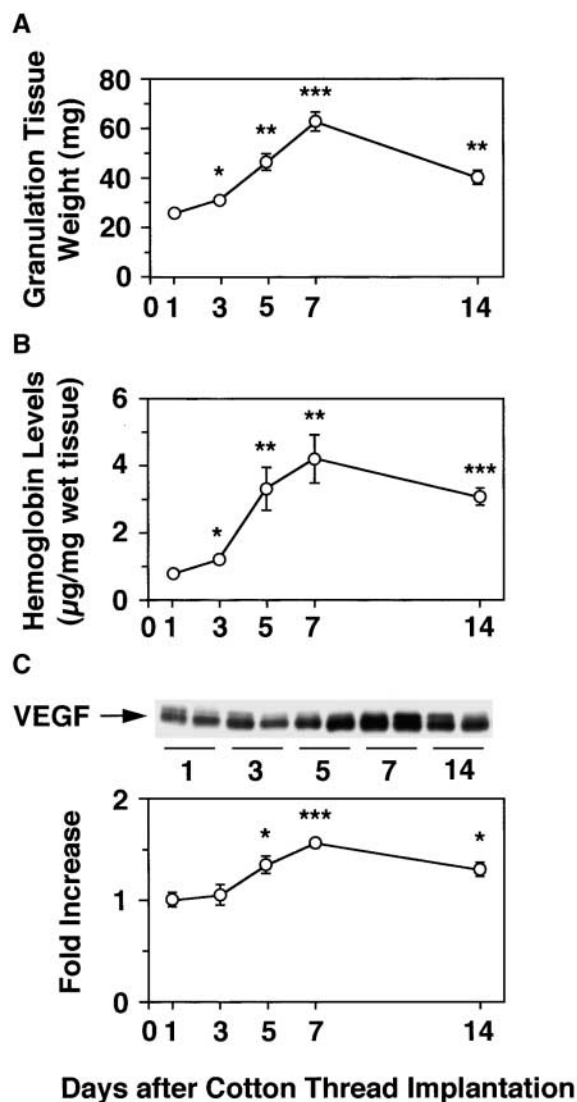
**Animals and Drugs.** HDC-deficient 129 inbred mice (25–28 g) were generated by a gene targeting method as described previously (24). WBB6F1<sup>+/+</sup> and WBB6F1-*W/W<sup>v</sup>* mice (25–28 g) were purchased from Japan SLC, Inc. Drugs used were pyrrolamine maleate, cimetidine (Sigma-Aldrich), thioperamide (a gift from J.C. Schwartz, Unite de Neurobiologie et Pharmacologie Moléculaire [U.109] de l'INSERM, Paris, France), dimaprit (Research Biochemicals International), histamine (Sigma-Aldrich), goat anti-VEGF IgG, and control goat IgG (R&D Systems).

**Induction of Cotton Thread-induced Granulation Tissue in Mice.** A cotton thread (model no. 8; Araiwa Co.) was washed overnight with ethyl acetate, and dried at room temperature before being cut into 1 cm in length (7 mg weight) and sterilized by dry heat at 160°C for 2 h. Male 129 inbred mice (25–28 g) were anesthetized and a cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum using a 13-G implant needle (Natsume Co.) according to the procedure described elsewhere (25) with a slight modification. The mice were treated in accordance with procedures approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan.

**Determination of Granulation Tissue Weight and Assessment of Angiogenesis in the Granulation Tissue.** Mice were anesthetized and killed by dislocation of the cervical spine at the indicated days after cotton thread implantation. The granulation tissue formed was dissected together with the cotton thread and weighed. Angiogenesis was assessed by taking photographs of the vascular network formation around the cotton thread and the subcutaneous tissue beneath the cotton thread. The dissected granulation tissue together with the cotton thread was washed in PBS, pH 7.4, and cut into small pieces with scissors before being homogenized in 20 vol of 0.5 mM sodium hydroxide using a Vir-Tis 45 homogenizer (Virtis Company) for 4 min at the scale 40 on an ice bed. The tissue homogenate was centrifuged at 10,000 *g* and 4°C for 30 min. An aliquot (200  $\mu$ l) of the supernatant was centrifuged again at 14,000 *g* and 4°C for 30 min. The hemoglobin concentration in the supernatant was then determined spectrophotometrically by measuring the absorbance at 540 nm using a hemoglobin assay kit (Hemoglobin B Test Wako; Wako Pure Chemical Ind.). The amount of hemoglobin in the granulation tissue was expressed as  $\mu$ g hemoglobin/mg wet tissue.

**Western Blot Analysis of VEGF Proteins.** After protein levels in the supernatant of the homogenate of the granulation tissue were determined (26), aliquots containing 0.56  $\mu$ g of protein were separated by electrophoresis on a 12% (wt/vol) SDS-poly-

acrylamide gel and then transferred onto a nitrocellulose membrane (Schleicher and Schuell, Inc.). The membrane was first incubated with mouse monoclonal anti-VEGF (1:200; Santa Cruz Biotechnology, Inc.) at 4°C for 12 h, followed by biotinylated anti-mouse IgG (1:2,000; Vector Laboratories) at 4°C for 3 h, and finally in the avidin-biotin-peroxidase complex (Vector Laboratories) at room temperature for 30 min. The reaction product was visualized with an ECL kit (ECL System; Amersham Pharmacia Biotech).



**Figure 1.** Granulation tissue formation by implantation of a cotton thread. A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of each mouse. The mice were killed 1, 3, 5, 7, and 14 d after cotton thread implantation. (A) The granulation tissue weight. (B) Hemoglobin levels in the granulation tissue. (C) VEGF protein levels in the granulation tissue. VEGF protein levels in the granulation tissue were determined by immunoblotting and analyzed densitometrically. Representative immunoblots from two mice in each group are shown at the top of C. The mean VEGF protein level in the granulation tissue 1 d after cotton thread implantation in the control group is set to 1.0. Values are the means from five to six mice with SEM shown by vertical bars. \* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\* $P < 0.001$  compared with values at 1 d after cotton thread implantation.

**Determination of VEGF Proteins.** Concentrations of VEGF proteins in the supernatant of the homogenate of the granulation tissue were determined using a commercially available ELISA kit (R&D Systems). In brief, 50  $\mu$ l of the assay diluent and 50  $\mu$ l of the diluted sample solution were incubated at room temperature in the dark for 2 h in each well of 96-well microplates precoated with polyclonal antibody specific to mouse VEGF. After washing, 100  $\mu$ l solution of an antibody against mouse VEGF conjugated to horseradish peroxidase was added to each well and incubated at room temperature in the dark for 2 h.

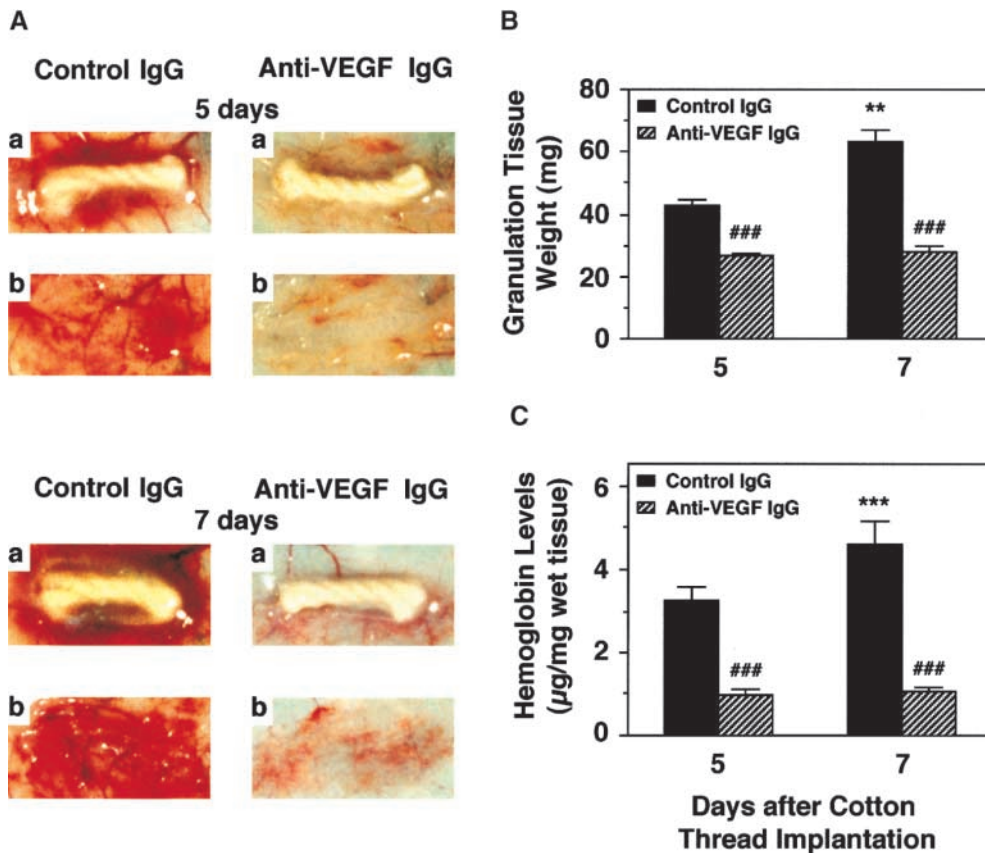
After incubation, the plates were washed again and 100  $\mu$ l of the substrate solution containing stabilized hydrogen peroxide and tetramethylbenzidine was added to each well and incubated for 30 min at room temperature in the dark. Thereafter, 100  $\mu$ l of the stop solution was added to each well and the absorbance at 450 nm was determined. The intensity of the colored product is proportional to the concentration of mouse VEGF present in the original samples.

Coefficient of variations in intraassay and interassay precision of this assay kit is 4.3–8.2% and 5.7–8.4%, respectively. Total protein contents in the supernatant of the homogenate of the granulation tissue were determined (26), and the contents of VEGF proteins were expressed as pg VEGF per  $\mu$ g total protein.

**Measurement of HDC Activity in the Tissue around the Cotton Thread.** A piece of the tissue, 2 cm in diameter surrounding the implanted cotton thread including the skin, cutaneous muscle layer, subcutaneous tissues, and the granulation tissue, was dissected together with the cotton thread, 0, 1, 3, and 5 d after cotton thread implantation. The dissected tissue including the cotton thread was homogenized in 20 vol of an HDC reaction buffer (0.1 M potassium-phosphate buffer, pH 6.8, 0.2 mM

dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml pepstatin, and 1% [vol/vol] polyethylene glycol, MW 300) for 4 min at the scale 40 on an ice bed using a Vir-Tis 45 homogenizer. The tissue homogenate was centrifuged at 10,000 g and 4°C for 30 min. The supernatant of the homogenate was then dialyzed overnight against the same buffer to remove endogenous histamine. Next, 1 ml of the dialyzed supernatant was preincubated at 37°C for 10 min. The sample was then incubated with or without L-histidine at 37°C for 3 h. The reaction was stopped by adding perchloric acid. Final concentrations of L-histidine and perchloric acid in the reaction mixture were 0.25 mM and 0.4 N, respectively. After centrifugation at 220 g and 4°C for 3 min, the supernatant was collected, and the amount of histamine in the supernatant determined fluorometrically (27). Protein levels in the dialyzed supernatant were determined (26), and HDC activity was expressed as the amount of histamine formed per min per mg protein.

**Immunostaining for HDC.** After dissection, the granulation tissue together with the cotton thread were fixed in PBS containing 10% (vol/vol) formalin for 72 h at 4°C. They were then dehydrated through three changes of 70, 80, 90, and 95% (vol/vol) ethanol, two changes of absolute ethanol and two changes of pure chloroform each 12 h. The samples were then embedded in paraffin and cut into sections. The sections (5  $\mu$ m) were then mounted on glass slides and spread by warming at 60°C for 30 min. The tissue sections were then deparaffined with xylene and ethanol, treated with 0.3% (vol/vol) hydrogen peroxide in methanol to inactivate endogenous peroxidase activity, and finally incubated in PBS containing 3% (wt/vol) BSA (Sigma-Aldrich) at room temperature for 1 h to block nonspecific staining. The slides were then incubated with rabbit polyclonal anti-HDC (1:750; Euro-

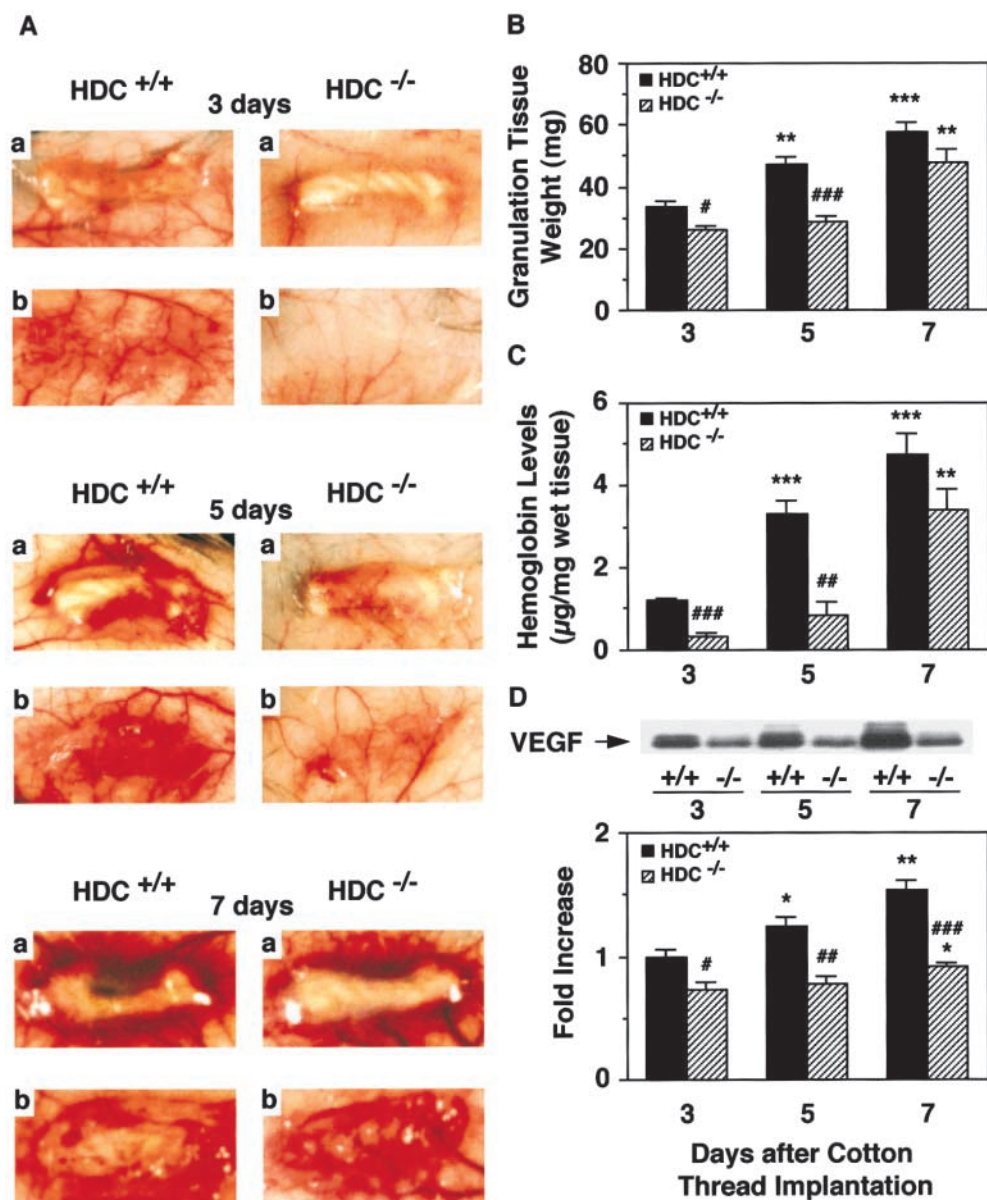


**Figure 2.** Effects of anti-VEGF antibody on angiogenesis and granulation tissue formation. A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of each mouse. Goat anti-VEGF IgG (1  $\mu$ g) or control goat IgG (1  $\mu$ g) dissolved in 100  $\mu$ l sterile PBS was subcutaneously injected at the site of cotton thread implantation just after the implantation and then once a day on consecutive days. The mice were killed 5 and 7 d after cotton thread implantation. (A) The vascular network formation around the cotton thread (a) and the subcutaneous tissue beneath the cotton thread (b). (B) The granulation tissue weight. (C) Hemoglobin levels in the granulation tissue. Values are the means from five mice with SEM shown by vertical bars. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared with values at 5 d in control group. ### $P < 0.001$  compared with values in control goat IgG treated group at corresponding days.

diagnostica) in 3% (wt/vol) BSA-PBS at 4°C for 1 h. After being washed with PBS, they were incubated with biotinylated anti-rabbit IgG (1:2,000; Vector Laboratories) in 3% (wt/vol) BSA-PBS at 4°C for 1 h and then with the avidin-biotin peroxidase complex (1:500; Vector Laboratories) in 3% (wt/vol) BSA-PBS at room temperature for 30 min. The reaction product was detected with 50 mM Tris-HCl buffer (pH 7.4) containing 0.05% (wt/vol) 3,3'-diaminobenzidine tetrahydrochloride (Dojin Chemical Laboratories), 0.04% (wt/vol) nickel chloride, and 0.033% (vol/vol) hydrogen peroxide and HDC-expressing cells were observed with a light microscope (original magnification:  $\times 400$ ).

**Immunohistochemical Observation for CD31 Expression.** After dissection, the granulation tissue together with the cotton thread was fixed, dehydrated, and embedded in paraffin as described above. The sections (5  $\mu$ m) were then mounted on glass slides and spread by warming at 60°C for 30 min. The tissue sections were then deparaffined with xylene and ethanol, treated with 0.3% (vol/vol) hydrogen peroxide in methanol to inactivate en-

dogenous peroxidase activity, and incubated in PBS containing 1.5% (wt/vol) blocking serum (Santa Cruz Biotechnology, Inc.) at room temperature for 1 h to block nonspecific staining. The slides were then incubated with goat polyclonal anti-CD31 (2  $\mu$ g/ml; Santa Cruz Biotechnology, Inc.) in PBS containing 1.5% (wt/vol) blocking serum at room temperature for 1 h. After being washed with PBS, they were incubated with biotinylated anti-goat IgG (1  $\mu$ g/ml; Santa Cruz Biotechnology, Inc.) in PBS containing 1.5% (wt/vol) blocking serum at room temperature for 1 h and then with the avidin-biotin peroxidase complex (1:500; Santa Cruz Biotechnology, Inc.) in PBS at room temperature for 30 min. The reaction product was detected with freshly prepared peroxidase substrate mixture containing 1.6 ml distilled water, five drops 10 $\times$  substrate buffer, 1 drop 50  $\times$  3,3'-diaminobenzidine tetrahydrochloride chromogen, and 1 drop 50 $\times$  peroxidase substrate (Santa Cruz Biotechnology, Inc.). CD31-expressing cells were observed with a light microscope (original magnification:  $\times 200$ ).



**Figure 3.** The defective angiogenesis in HDC<sup>-/-</sup> mice. A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of each mouse. The mice were killed 3, 5, and 7 d after cotton thread implantation. (A) The vascular network formation around the cotton thread (a) and the subcutaneous tissue beneath the cotton thread (b). (B) The granulation tissue weight. (C) Hemoglobin levels in the granulation tissue. (D) VEGF protein levels in the granulation tissue. VEGF protein levels in the granulation tissue were determined by immunoblotting and analyzed densitometrically. Representative immunoblots from one mouse in each group are shown at the top of D. The mean VEGF protein level in the granulation tissue 3 d after cotton thread implantation in HDC<sup>+/+</sup> mice is set to 1.0. Values are the means from five mice with SEM shown by vertical bars. \* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\* $P < 0.001$  compared with values at 3 d in HDC<sup>+/+</sup> mice. # $P < 0.05$ ; ## $P < 0.01$ ; and ### $P < 0.001$  compared with values in HDC<sup>+/+</sup> mice at corresponding days.

**Statistical Analysis.** Statistical significance of the results was analyzed by Dunnett's test for multiple comparisons and Student's *t* test for unpaired observations.

## Results

**Cotton Thread-induced Granulation Tissue Formation, Angiogenesis, and VEGF Production.** Subcutaneous implantation of a cotton thread in the dorsum of HDC<sup>+/+</sup> mice induced gradual increases in the weight and hemoglobin levels of the granulation tissue from 3 to 7 d after cotton thread implantation (Fig. 1 A and B). In addition, the vascular network formation of the granulation tissue and subcutaneous tissue beneath the cotton thread also increased over the same time points (Figs. 2 A and 3 A). The levels of VEGF protein in the granulation tissue were also increased (Fig. 1 C). To clarify the role of VEGF in the angiogenesis and granulation tissue formation, goat anti-VEGF IgG or control goat IgG was subcutaneously injected at the site of cotton thread implantation just after the implantation and once a day on consecutive days. The injection of goat anti-VEGF IgG, but not control goat IgG, suppressed the vascular network formation, as well as the increases in hemoglobin levels and granulation tissue weight 5–7 d after cotton thread implantation (Fig. 2). These findings suggest that the cotton thread-induced angiogenesis is highly dependent on VEGF and that the inhibition of angiogenesis results in the inhibition of granulation tissue formation.

**Defective Angiogenesis in HDC<sup>-/-</sup> Mice.** To clarify the role of histamine in angiogenesis, we implanted a cotton thread in the dorsum of HDC<sup>-/-</sup> mice. Vascular network formation in the granulation tissue and the subcutaneous tissue beneath the cotton thread in HDC<sup>-/-</sup> mice 3 and 5 d after cotton thread implantation was notably less than those in HDC<sup>+/+</sup> mice (Fig. 3 A). In addition, the granulation tissue formation (Fig. 3 B), as well as the levels of hemoglobin (Fig. 3 C) and VEGF protein in the granulation tissue (Fig. 3 D) of HDC<sup>-/-</sup> mice were significantly less

**Table I.** VEGF Protein Levels in the Supernatant of the Homogenate of the Cotton Thread-induced Granulation Tissue

| Mice               | Days after implantation | VEGF protein levels <sup>a</sup> |
|--------------------|-------------------------|----------------------------------|
| HDC <sup>+/+</sup> | 3                       | 0.27 ± 0.02                      |
|                    | 5                       | 0.37 ± 0.03 <sup>b</sup>         |
|                    | 7                       | 0.42 ± 0.02 <sup>c</sup>         |
| HDC <sup>-/-</sup> | 3                       | 0.17 ± 0.02 <sup>d</sup>         |
|                    | 5                       | 0.19 ± 0.02 <sup>e</sup>         |
|                    | 7                       | 0.24 ± 0.03 <sup>a, e</sup>      |

<sup>a</sup>VEGF protein levels are expressed as pg VEGF per µg total protein. Data represent mean ± SEM from four mice.

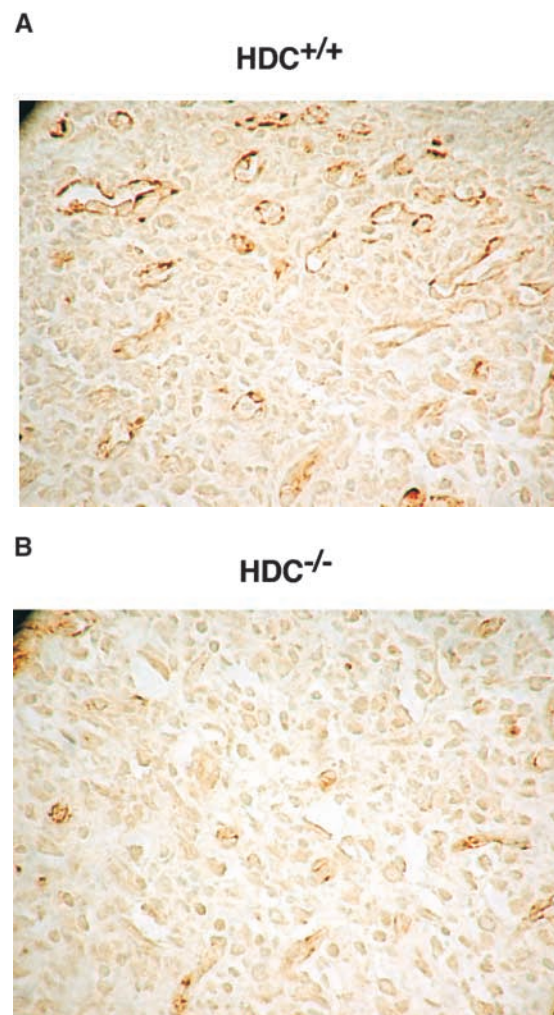
<sup>b</sup>*P* < 0.05.

<sup>c</sup>*P* < 0.01 compared with values in corresponding 3 d group.

<sup>d</sup>*P* < 0.05.

<sup>e</sup>*P* < 0.01 compared with values in HDC<sup>+/+</sup> mice at corresponding days.

than those in HDC<sup>+/+</sup> mice 3 and 5 d after cotton thread implantation. Concentrations of VEGF proteins in the supernatant of the homogenate of the cotton thread induced-granulation tissue determined by ELISA also revealed that the levels of VEGF proteins in the granulation tissue 3 to 7 d after cotton thread implantation were lower in HDC<sup>-/-</sup> mice than in HDC<sup>+/+</sup> mice (Table I). In addition, immunohistochemical analysis of the granulation tissue dissected 5 d after the cotton thread implantation using an antibody to CD31, a marker for endothelial cells, indicated that the neovascular density in the granulation tissue in HDC<sup>-/-</sup> mice was obviously less than that in HDC<sup>+/+</sup> mice (Fig. 4). These findings indicate that histamine plays an important role in the induction of VEGF and angiogenesis during the early stage of cotton thread-induced granulation tissue.

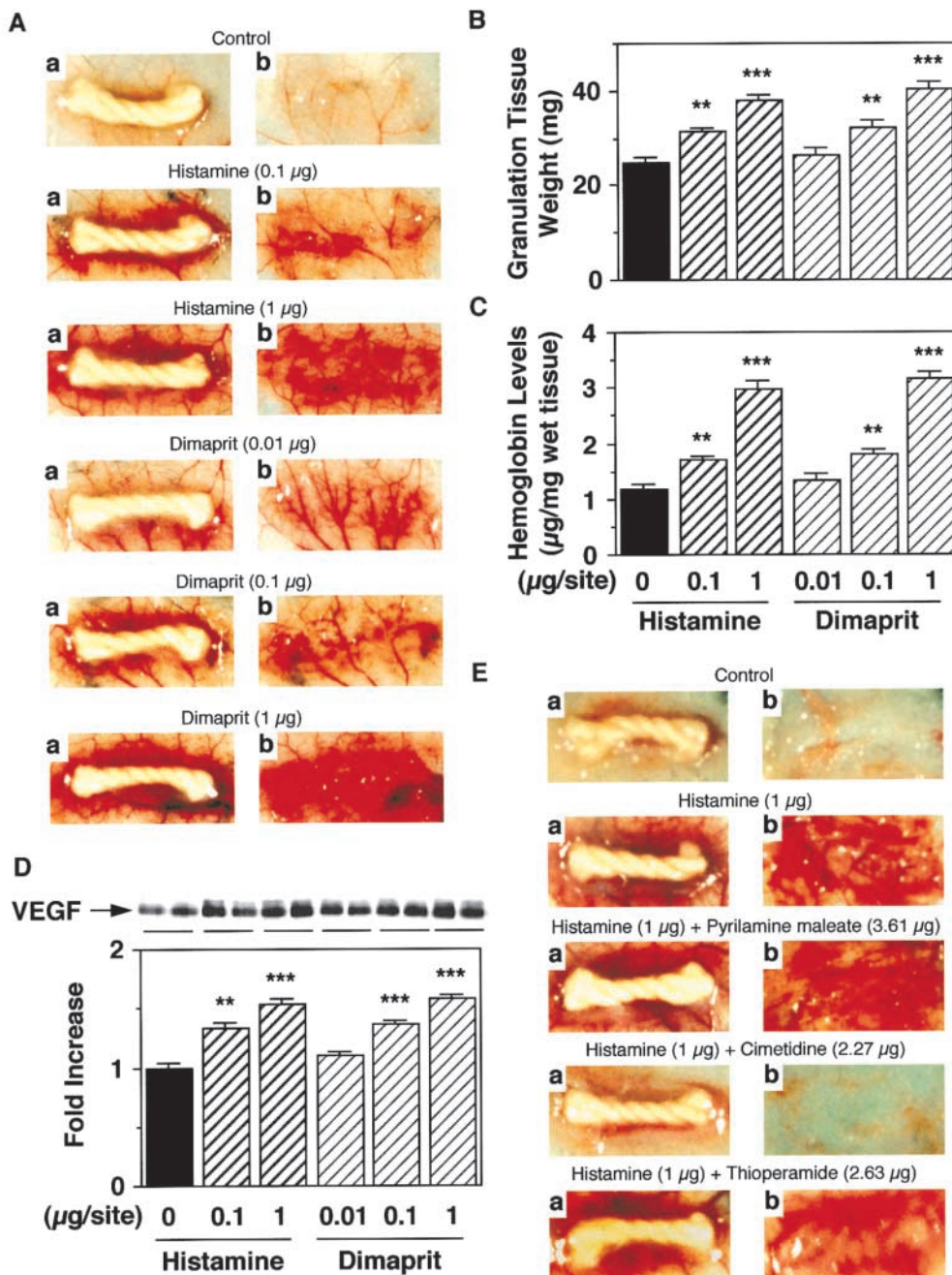


**Figure 4.** Immunohistochemical observation for CD31 expression in the granulation tissue in mice. A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of each mouse. The mice were killed 5 d after cotton thread implantation. The sections of the granulation tissue were immunostained using an antibody to CD31, a marker for endothelial cells. CD31 expression in the granulation tissue in HDC<sup>+/+</sup> mice (A) and in HDC<sup>-/-</sup> mice (B) was observed with a light microscope (original magnification: ×200). Representative micrographs are shown from three samples.

*Recovery of Defective Angiogenesis in HDC<sup>-/-</sup> Mice by Histamine and Dimaprit.* Subcutaneous injection of histamine (0.1 and 1  $\mu\text{g}$ ) or the H<sub>2</sub> receptor agonist dimaprit (0.01, 0.1, and 1  $\mu\text{g}$ ) at the site of implantation just after the implantation and then once a day on consecutive days significantly enhanced angiogenesis in the granulation tissue as well as the granulation tissue formation itself compared with those in mice treated with saline (Fig. 5 A–C). The VEGF level in the granulation tissue was also increased by the injection of histamine or dimaprit in a dose-dependent manner (Fig. 5 D). In addition, topical injections of the H<sub>2</sub> receptor antagonist cimetidine inhibited

the histamine-induced angiogenesis in the granulation tissue and subcutaneous tissue beneath the cotton thread (Fig. 5 E). In contrast, the H<sub>1</sub> receptor antagonist pyrilamine maleate and the H<sub>3</sub> receptor antagonist thioperamide had no effect. Cimetidine also inhibited the histamine-induced increases in granulation tissue weight, and levels of hemoglobin and VEGF in the granulation tissue (data not shown). These findings indicate that histamine augments VEGF production via H<sub>2</sub> receptors.

*Involvement of Histamine Derived from Nonmast Cells in Angiogenesis.* To clarify whether the histamine responsible for angiogenesis was derived from mast cells, we compared



**Figure 5.** Effects of histamine and dimaprit on angiogenesis in HDC<sup>-/-</sup> mice. A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of each mouse. Histamine (0, 0.1, and 1  $\mu\text{g}$ ), pyrilamine maleate (3.61  $\mu\text{g}$ ), cimetidine (2.27  $\mu\text{g}$ ), thioperamide (2.63  $\mu\text{g}$ ), or dimaprit (0.01, 0.1, and 1  $\mu\text{g}$ ) dissolved in 100  $\mu\text{l}$  sterile saline were injected subcutaneously at the site of cotton thread implantation just after the implantation and then once a day on consecutive days. The mice were killed 5 d after cotton thread implantation. (A and E) The vascular network formation around the cotton thread (a) and the subcutaneous tissue beneath the cotton thread (b). (B) The granulation tissue weight. (C) Hemoglobin levels in the granulation tissue. (D) VEGF protein levels in the granulation tissue. VEGF protein levels in the granulation tissue were determined by immunoblotting and analyzed densitometrically. Representative immunoblots from two mice in each group are shown at the top of D. The mean VEGF protein level in the granulation tissue in control mice is set to 1.0. Values are the means from five mice with SEM shown by vertical bars. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared with values in control group.

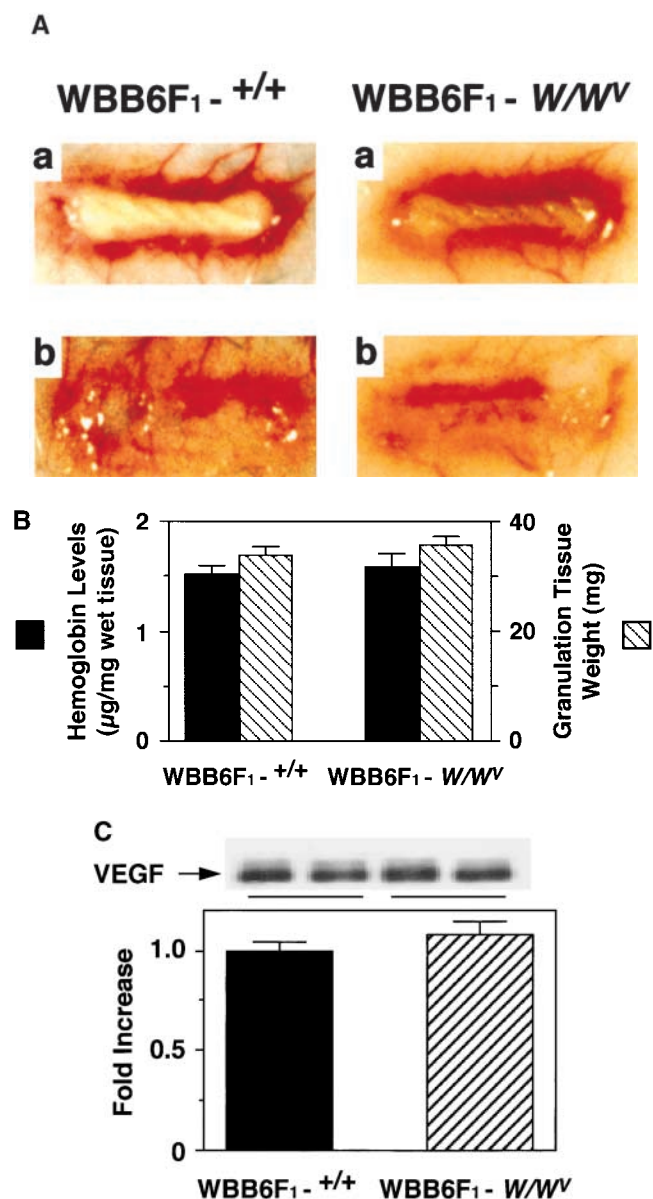
the cotton thread-induced granulation tissue formation and angiogenesis in mast cell-deficient mice ( $WBB6F_1-W/W^V$ ) with those in wild-type mice ( $WBB6F_1^{+/+}$ ). We found that there was no significant difference in the granulation tissue formation, angiogenesis and VEGF levels in the granulation tissue between the two groups (Fig. 6). In  $HDC^{+/+}$  mice, the implantation of a cotton thread increased the HDC activity in the tissue surrounding the implanted cotton thread including the skin, cutaneous muscle layer, subcutaneous tissues and the granulation tissue, from 1 to 5 d after implantation (Fig. 7 A). In  $WBB6F_1-W/W^V$  mice, although the HDC activity in the tissue was very low due to the mast cell deficiency, the cotton thread implantation induced a marked increase in HDC activity in the tissue to a similar extent as that in  $WBB6F_1^{+/+}$  mice (Fig. 7 B). In addition, histochemical analysis of the granulation tissue dissected 5 d after the cotton thread implantation indicated the absence of mast cells in the tissue (data not shown). However, the HDC-producing cells were shown to be infiltrating macrophages (Fig. 7 C). These findings indicate that HDC expression is induced in the nonmast cell, macrophages.

## Discussion

Histamine plays a variety of roles as an autacoid which regulates allergic inflammatory reactions (18, 28), differentiation of leukocytes precursors (29), neutrophil infiltration into the inflammatory sites (17), and gastric acid secretion (30). It also acts as a neurotransmitter in the central nervous system (31). In this study, we demonstrated a significant role of histamine in angiogenesis, i.e., histamine enhances angiogenesis in the inflammatory granulation tissue via the induction of VEGF production.

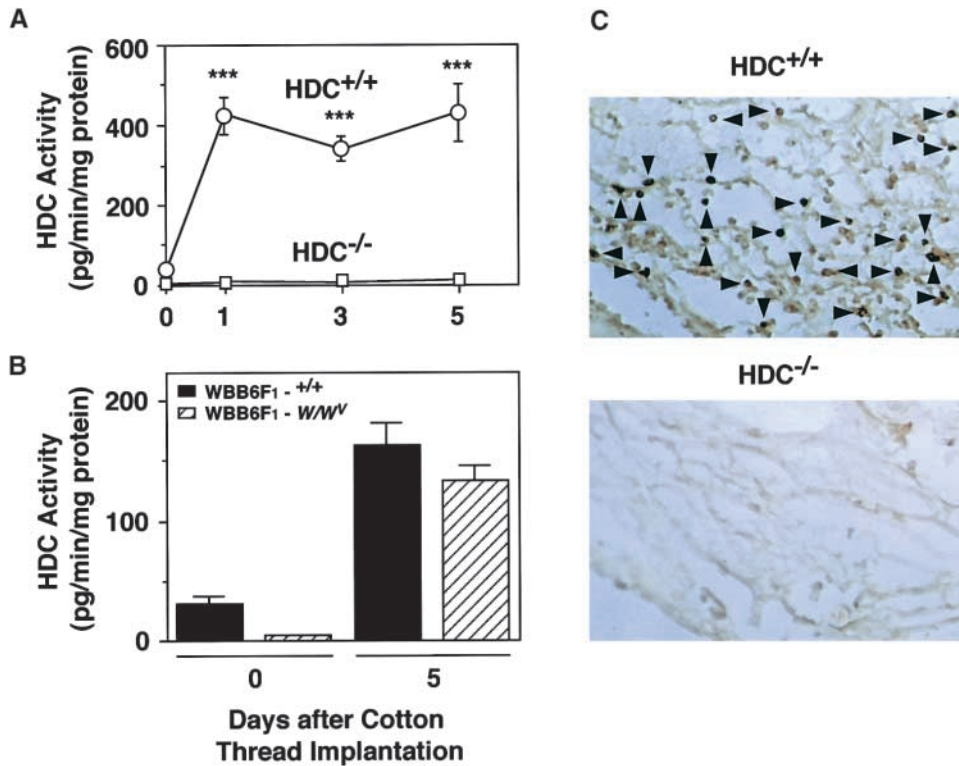
Subcutaneous implantation of a cotton thread induced rapid formation of granulation tissue together with angiogenesis (Figs. 1 and 3). The angiogenesis was highly dependent on VEGF production, as it was strongly inhibited by goat anti-VEGF IgG (Fig. 2). In addition, we also demonstrated that a reduction in the granulation tissue formation was accompanied by the inhibition of angiogenesis (Fig. 1). Thus, the mechanism of angiogenesis in the proliferative inflammatory tissue can be studied *in vivo* by using the mouse model of inflammation induced by cotton thread implantation. Using  $HDC^{-/-}$  mice, we found that the development of angiogenesis especially in the early phase (3–5 d after cotton thread implantation) depends on endogenous histamine (Figs. 3 and 4). The possibility that the functioning of hemangioblasts and endothelial cells were defective due to the destruction of the HDC gene was ruled out because the injection of histamine or dimaprit rescued the defective angiogenesis in  $HDC^{-/-}$  mice (Fig. 5).

The production of VEGF in  $HDC^{-/-}$  mice was significantly less than  $HDC^{+/+}$  mice, and the injection of dimaprit or histamine increased the VEGF levels in the granulation tissue (Fig. 5 D). In addition, topical injection of



**Figure 6.** Comparison of granulation tissue formation between  $WBB6F_1^{+/+}$  and  $WBB6F_1-W/W^V$  mice. A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of each mouse. The mice were killed 5 d after the cotton thread implantation. (A) The vascular network formation around the cotton thread (a) and the subcutaneous tissue beneath the cotton thread (b). (B) The granulation tissue weight and hemoglobin levels in the granulation tissue. (C) VEGF protein levels in the granulation tissue. VEGF protein levels in the granulation tissue were determined by immunoblotting and analyzed densitometrically. Representative immunoblots from two mice in each group are shown at the top of C. The mean VEGF protein level in the granulation tissue of  $WBB6F_1^{+/+}$  mice is set to 1.0. Values are the means from five mice with SEM shown by vertical bars.

cimetidine inhibited the histamine-induced angiogenesis in  $HDC^{-/-}$  mice (Fig. 5 E). Therefore, we concluded that endogenous histamine enhances VEGF production via  $H_2$  receptors. This conclusion corresponds with our previous *in vitro* findings that exogenous histamine induces VEGF production by macrophages, endothelial cells, and fibro-



**Figure 7.** HDC activity in the tissue surrounding the implanted cotton thread. A cotton thread (1 cm, 7 mg) was implanted subcutaneously in the dorsum of each mice. (A) HDC<sup>+/+</sup> and HDC<sup>-/-</sup> mice were killed and a piece of the tissue, 2 cm in diameter surrounding the implanted cotton thread including the skin, cutaneous muscle layer, subcutaneous tissues, the granulation tissue together with the cotton thread, was dissected 0, 1, 3, and 5 d after cotton thread implantation. The dissected tissue and cotton thread were homogenized and the HDC activity in the supernatant was determined. Values are the means from five to six mice with SEM shown by vertical bars. \*\*\**P* < 0.001 compared with the HDC activity in the tissue dissected just after cotton thread implantation in HDC<sup>+/+</sup> mice. (B) WBB6F1<sup>+/+</sup> and WBB6F1<sup>-W/W<sup>V</sup></sup> mice were killed and a piece of the tissue, 2 cm in diameter surrounding the implanted cotton thread including the skin, cutaneous muscle layer, subcutaneous tissues, the granulation tissue together with the cotton thread, was dissected 0 and 5 d after cotton thread implantation. The dissected

tissue and cotton thread were homogenized and the HDC activity in the supernatant was determined. (C) HDC-expressing cells in the granulation tissue dissected 5 d after cotton thread implantation in HDC<sup>+/+</sup> and HDC<sup>-/-</sup> mice were detected immunohistochemically using rabbit polyclonal anti-HDC, and observed with a light microscope (original magnification: ×400). HDC-producing cells are mainly macrophages and are indicated by closed arrowheads. Representative micrographs are shown from four samples.

blasts present in the granulation tissue via the H<sub>2</sub> receptor-cAMP-protein kinase A pathway (23).

Although it is known that there is increased histamine production in rapidly growing tissues (16, 17), the histamine-producing cells have not yet been identified. It has been reported that the number of mast cells in the rapidly growing tissues increases (32) and that mast cell-derived histamine is angiogenic (33). However, we found that HDC activity in the tissue surrounding the implanted cotton thread including the skin, cutaneous muscle layer, subcutaneous tissues, and the granulation tissue increased even in WBB6F1<sup>-W/W<sup>V</sup></sup> mice (Fig. 7 A and B). In addition, histochemical analysis of the granulation tissue dissected 5 d after the cotton thread implantation indicated the absence of mast cells in the granulation tissue (data not shown). In contrast, HDC-producing cells in the granulation tissue were identified as infiltrating macrophages (Fig. 7 C). Histamine production by nonmast cells was observed in mouse skin treated with PMA (34), in various tissues such as liver and lung in IL-1-treated mice (35), and in the infiltrating leukocytes in allergic inflammation in rats (36). Our findings did not exclude the possibility that mast cells in the granulation tissue and the surrounding tissues release histamine.

Because the cotton thread implantation induced an apparent angiogenesis in WBB6F1<sup>-W/W<sup>V</sup></sup> mice as well as in WBB6F1<sup>+/+</sup> mice, we concluded that histamine from infil-

trating macrophages play a significant role in angiogenesis of the granulation tissue. It is reported that cimetidine, an H<sub>2</sub> antagonist, delays wound healing especially in peptic ulcer (37, 38), but its mechanism of action has not been clarified. We found that cimetidine reduces VEGF production in the carrageenin-induced inflammation model in rats (23). In the cotton thread-induced inflammation model in mice, treatment with cimetidine showed partial but significant inhibition of angiogenesis and VEGF production (data not shown). Therefore, we hypothesize that cimetidine delays wound healing by inhibiting histamine-mediated VEGF production and angiogenesis. Our findings also suggest that H<sub>2</sub> receptor antagonists would be useful for suppressing the angiogenesis-dependent formation of inflammatory granulation tissue, and conversely, that H<sub>2</sub> receptor agonists may enhance angiogenesis in wound healing and several ischemic diseases. In addition, because cimetidine inhibits tumor growth in vivo (39, 40), there is a possibility that the growth of some tumors may also be regulated by histamine-mediated angiogenesis.

These findings demonstrate that the defective angiogenesis in the inflammatory granulation tissue is induced in HDC<sup>-/-</sup> mice but not in mast cell-deficient mice, and histamine derived from nonmast cells plays a significant role in the angiogenesis of the inflammatory granulation tissue.

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